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82. Kazukichi Kato : A New Color Reaction of Steroid with Anhydrous Aluminum Chloride and Anisaldehyde. II.*1 Application to the Colorimetric Determination of Cholesterol and Selectivity of the Reaction.

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There are numerous reports on the color reaction of cholesterol, and the methods with sulfuric acid-acetic anhydride (Liebermann-Burchard's reaction),[1,2] with sulfuric acid-acetic acid-ferric chloride (Zlatkis-Zak's reaction),[3] and with zinc chloride-acetyl chloride (Tschugaeff's reaction),[4] these are applied to its colorimetric determination.

In the previous paper, a new colorimetric determination of ethylestrenol with anhydrous aluminum chloride and anisaldehyde was reported. These reagents give a sensitive and stable reddish violet color also with cholesterol, and were successfully applied to its colorimetric determination. In order to define the selectivity of this color reaction, scores of steroids and other compounds were examined under the analogous condition. In the course of the survey, it was found that cyclohexanol gives also a sensitive violet color, which resembles that obtained with concentrated sulfuric acid and benzaldehyde in its Komarowsky's reaction.[5] Accordingly, the reaction course of cyclohexanol with anhydrous aluminum chloride and anisaldehyde was analysed by gas chromatography, and it was confirmed that there are a clear difference between the mechanism of this new reaction and of Komarowsky's.

I. Colorimetric Determination of Cholesterol

In the application of this new reaction to the assay of cholesterol, it is remarked that a higher absorbance is obtained by setting some interval between the addition of anhydrous aluminum chloride and of anisaldehyde, as discussed below. Such an effect was not found in case of ethylestrenol.

Experimental

Material——Cholesterol : Cholesterol (J. P.) was dried at 80° to constant weight under reduced pressure (5 mm. Hg), m.p. 148.5°. *Anal.* Calcd. for $C_{27}H_{46}O$: C, 83.87; H, 11.99. Found : C, 83.77; H, 11.84.

Sample Solution——An accurately weighed quantity of cholesterol was dissolved in $CHCl_3$ to prepare a sample solution. Unless otherwise described, the sample solution in the following experiments contained 100 μg. of cholesterol per 1 ml.

The test solutions were prepard in accordance with the same procedure, and the measurements were carried out with the same apparatus, as described in the preceding paper.

Procedure——To 1 ml. of the sample solution containing 50~200 μg. of cholesterol in a 10 ml. volumetric flask, 1 ml. of 5% anhyd. $AlCl_3$ solution was added, and shaken thoroughly. Being tightly stoppered, it was heated in a water bath at 50° for 1 hr. After cooled rapidly to 20~30°, 1 ml. of 5% anisaldehyde solution was added, and the colored solution was allowed to stand at 20~30° for 50 min. Then it was diluted to

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*1 Part I : This Bulletin, **12**, 578 (1964).

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1) C. Liebermann : Ber., **18**, 1803 (1885).
2) H. Burchard : Chem. Zetr., **61**, (I), 25 (1890).
3) B. Zak, N. Moss, A. J. Boyle, A. Zlatkis : Anal. Chem., **26**, 776 (1954).
4) L. Tschugaeff : Chemiker Ztg., **24**, 542 (1900).
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10 ml. with nitrobenzene-benzene (1:1) solution, and its absorbance at 536 mμ was measured within 15 min. As a blank, 1 ml. of $CHCl_3$ was operated in the same manner, to be referred to the sample solution in the measurement.

Discussion

Absorption Spectrum of Colored Solution

The colored solution, which was obtained from 1 ml. of the sample solution by the above procedure, gave an absorption spectrum, as shown in Fig. 1, and its absorption maximum exists at 536 mμ. The absorbance of the blank at this wave length is about 0.04. Therefore, the absorbance of the sample solutions was measured at this wave length.

Calibration Curve

The sample solutions, which contained 25~200 μg. of cholesterol per 1 ml. respectively, were operated by the above procedure, and a calibration curve was obtained as shown in Fig. 2,

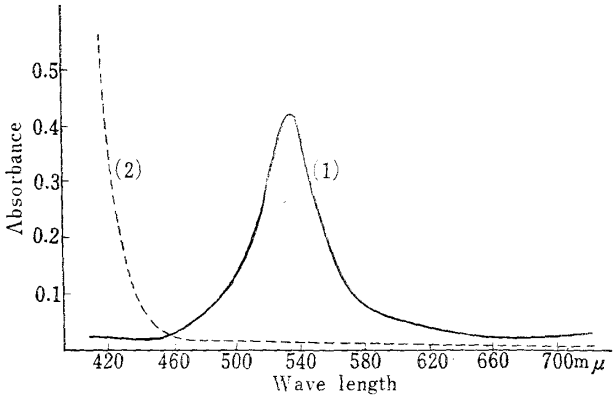

Fig. 1. Absorption Spectra of Colored Solution

(1): Colored solution obtained from 100 μg. of cholesterol, referred to blank solution.
(2): Blank solution

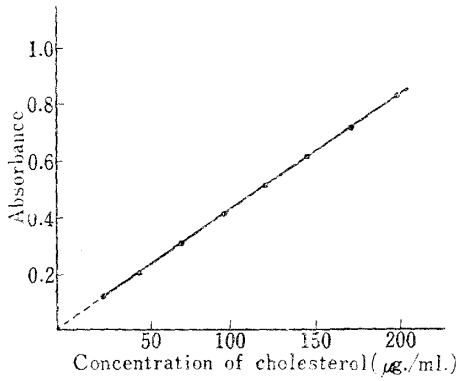

Fig. 2. Calibration Curve

Effect of the Concentrations of the Reagents

Results were obtained as shown in Table I, when the concentration of both reagents were varied, while other conditions were left as mentioned in the procedure of the colorimetric determination. While absorbance increases, as the concentration of anisaldehyde increases, or the concentration of anhydrous aluminum chloride decreases, the colored solution becomes turbid at lower concentration of anhydrous aluminum chloride, or at the higher concentration of anisaldehyde than those described in Table I. Therefore the determination was carried out with 5% anisaldehyde solution and 5% anhydrous aluminum chloride solution.

Table I. Effect of the Concentration of the Reagents on Absorbance

Concn. of $AlCl_3$ (%) / Concn. of anisaldehyde (%)	4.5	5.0	5.5	6.0	6.0
3.5	0.392	0.377	0.360	0.311	0.261
4.0	0.418	0.400	0.380	0.319	0.292
4.5	0.431	0.423	0.413	0.361	0.319
5.0	0.433	0.428	0.420	0.371	0.340
5.5	0.346	0.430	0.419	0.377	0.354

Effect of the Reaction Temperature and the Elapsed Time from the Addition of 5% Anhydrous Aluminum Chloride Solution to of 5% Anisaldehyde Solution on Absorbance

Results were obtained as shown in Table II, when the reaction temperature and reaction time after the addition of 5% anhydrous aluminum chloride solution were varied, while other conditions were left as described in the procedure of the colorimetric determination.

TABLE II. Effect of the Reaction Temperature and the Elapsed Time from the Addition of 5% Anhydrous Aluminum Chloride Solution to the Addition of 5% Anisaldehyde Solution on Absorbance

Elapsed time (min.) / Reaction temp. (°C)	20	40	60	80
60	0.419	0.431	0.431	0.428
50	0.415	0.425	0.428	0.426
40	0.379	0.399	0.423	0.426
30	0.328	0.345	0.368	0.388

Absorbance does not change in a range of temperature 50~60° and of reaction time 40~80 min. Because of the evaporation of chloroform at 60°, the reaction temperature of 50° and the reaction time of 60 min. were adopted for the determination procedure.

Effect of the Reaction Temperature and Elapsed Time after the Addition of 5% Anisaldehyde Solution

Results were obtained as shown in Table III, when the reaction temperature and reaction time after the addition of 5% anisaldehyde solution were varied, while other conditions were left as described in the determination procedure. Absorbance does not change in a range of temperature 20~40°, and of time 40~80 min. When the reaction temperature reaches to 40°, the absorbance of the blank is over 0.05. Therefore, the reaction temperature of 20~30° and the reaction time of 50 min. were adopted as the conditions of the determination.

TABLE III. Effect of the Reaction Temperature and the Elapsed Time after the Addition of 5% Anisaldehyde Solution on Absorbance

Elapsed time (min.) / Reaction temp. (°C)	10	20	40	50	60	80
40	0.377	0.429	0.425	0.425	0.423	0.422
30	0.333	0.423	0.428	0.428	0.429	0.427
20	0.314	0.388	0.425	0.425	0.428	0.423
10	0.306	0.317	0.349	0.387	0.409	0.422

Stability of the Colored Solution

Results were obtained as shown in Table IV, when the colored solution was allowed to stand for 30 min. after being diluted with nitrobenzene-benzene (1:1) solution.

Absorbance is unvaried within 15 min.

TABLE IV. Stability of the Colored Solution

Time after dilution (min.)	1	5	10	15	20	30
Absorbance	0.428	0.428	0.426	0.425	0.421	0.415

Error of the Assay

$\hat{\sigma}$ was 1.09% (n=6), calculated on the data, which were obtained from a sample solution containing 100 μg. of cholesterol per 1 ml., as shown in Table V.

Table V. Results on a Sample Solution containing 100 μg. of Cholesterol per 1 ml.

	1	2	3	4	5	6	$\bar{x}$
Absorbance	0.425	0.432	0.435	0.423	0.425	0.427	0.428

II. Selectivity and Other Characteristics of the Color Reaction

Selectivity of the Color Reaction

The selectivity and sensitivity of the reaction were investigated on scores of steroids and other compounds, depending on the reaction condition analogous to that of determination of cholesterol.

Each sample was dissolved in chloroform to prepare the sample solution. Upper limit of the concentration was 1 mg. per 1 ml. To 1 ml. of the sample solution, 1 ml. of 5% anhydrous aluminum chloride solution was added, and shaken thoroughly. After heating in a water bath at 50° for 20 min., it was cooled rapidly to 20~30°, and 1 ml. of 5% anisaldehyde solution was added. After 20 min., the colored solution was diluted with nitrobenzene-benzene (1:1) solution to 10 ml., and its absorption spectrum was measured by the autorecording spectrophotometer. In order to determine the limit of identification, each sample solution was diluted stepwise with chloroform, and operated in the same manner as mentioned above. Its coloration was observed with the naked eye.

On various steroids, results were obtained as shown in Table VI.

As for the relationship between the chemical structure and coloration, the following conclusions are drawn. i) The steroid, which has an isolated double bond or a double bonds conjugated between four carbon atoms in its nucleus, shows a nearly same coloration and sensitivity with those of cholesterol. ii) The saturated steroid, such as cholestane, and the steroid having one or two double bonds conjugated to a ketonic group do not show coloration. iii) Even the saturated steroid or the keto-conjugated unsaturated steroid gives a color with the sensitivity several times lower than the unsaturated steroid mentioned above, if it has a hydroxyl group easily dehydrated in other position of the molecule.

On the other hand, estrone and estradiol, both having a phenolic A ring, gave a red color with anhydrous aluminum chloride solution alone. Among unsaturated compounds except steroid, cinnamic acid, morphine, thebaine and codeine gave no color, even if 10 mg. of each sample was submitted to the test. But 1 mg. of cyclohexene and 10 mg. of oleic acid showed as good reddish violet and red coloration respectively, as 100 μg. of cholesterol. Cyclohexanol also gave the same reddish violet color as cyclohexene.

From a consideration of these results, it seems most reasonable to assume that the functional group responsible for the development of color is a type of double bond, which exists originally in the molecule, or which is produced by the elimination of a hydroxyl group in the course of the reaction. In all cases, it is a necessary condition that these double bonds do not conjugate with any carbonyl group.

Availability of Reagents

The combination of both reagents, *i.e.*, anhydrous aluminum chloride and anisaldehyde, was selected to be most suitable, after several metal halides and aromatic aldehydes were submitted to the test for cholesterol, combined with each other.

Table VI. Color Reaction of Steroids with Anhydrous Aluminum Chloride and Anisaldehyde

Steroid	Color[b]	λ_{max} (mμ)	Limit of identification (μg./ml.)
Cholest-4-ene	RV	540	10
Cholest-5-ene	〃	540	10
Cholest-3,5-diene	〃	536	5
α-Cholestanol	〃	545	30
β-Cholestanol	〃	545	30
Cholesterol	〃	536	5
Cholesteryl acetate	〃	536	5
Cholesteryl benzoate	〃	536	5
Cholesteryl palmitate	〃	536	5
Cholesteryl stearate	〃	536	5
Cholesteryl chloride	〃	536	5
Allocholesterol	〃	537	10
Ergosterol	V	557	5
Stigmasterol	RV	536	5
Pregnenolone	V	553	5
Pregnenolone acetate	〃	553	5
Dehydroepiandrosterone	〃	560	5
Dehydroepiandrosterone acetate	〃	560	15
3β-Acetoxy-B-nor-cholest-5-ene	RV	540	5
3β,16,17-Trihydroxy-6β-methyl-5-pregnene-20-one[a]	V	576	5
3β-Hydroxy-16,17-epoxy-6β-methyl-5-pregnene-20-one[a]	〃	570	5
Cholestane	negative		
3β-Acetoxy-cholest-5-ene-7-one	〃		
Cholest-3,5-diene-7-one	〃		
Cholest-4-ene-3-one	〃		
Testosterone	〃		
Methyltestosterone	RV	547	30
19-Nor-testosterone	negative		
19-Nor-testosterone phenylpropionate	〃		
Corticosterone	〃		
Hydrocortisone	〃		
Progesterone	〃		
Prednisolone	〃		
Cortexolone	〃		
11-Hydroxy-1,4-androstadiene-3,17-dione	〃		
17α-Hydroxyl-4,9(11)-pregnatriene-3,20-dione	〃		
Deoxycholic acid	RV	545	30

a) The author thanks Dr. V. Petrow, The British Drug Houses, Ltd., for supplying these samples.
b) RV: Reddish violet. V: Violet.

Zinc chloride gave no color, and antimony trichloride was far less sensitive than aluminum chloride, when they were used together with aldehydes. As to aldehyde, benzaldehyde, *p*-hydroxy-, *p*-nitro-, *p*-chloro-, *p*-dimethylamino-, *o*-hydroxy-, and *o*-nitro-benzaldehyde, and cinnamaldehyde, these showed no coloration in combination with anhydrous aluminum chloride. *m*-Hydroxy-, 2,3-dimethoxy-, 2,4-dimethoxy-, 3,4-dimethoxy-benzaldehyde gave themselves a red color with anhydrous aluminum chloride and vanillin caused turbidity.

With regard to the solvent of anhydrous aluminum chloride, chloroform, ether, benzene, nitromethane, and acetonitrile were also tested. In chloroform, ether and benzene the solubility of the halide is far smaller than in nitrobenzene, and the sample solution gave only a faint and unstable color, being turbid by the addition of anisaldehyde. By nitromethane or acetonitrile solution of anhydrous aluminum chloride,

color never developed, when anisaldehyde was added, in spite of the large solubility of the halide.

Recently, Becker used anhydrous aluminum chloride to distinguish 3-keto-4-ene-steroid from 3-keto-1, 4-diene,[6] and to determine estrogen,[7] but it has been never applied in combination with an aromatic aldehyde to a color reaction.

Aromatic aldehyde were used as reagent for qualitative detection of steroid by Kägi and Miescher,[8] who heated acetic acid solution of steroid with concentrated sulfuric acid and aldehyde. Their reaction, however, is specific for the steroid having a secondary α-hydroxyl group at its 17-position, and gives a negative result for cholesterol. While benzaldehyde and concentrated sulfuric acid are used for the detection of several aliphatic unsaturated compounds, such as morphine alkaloids,[9] anhydrous aluminum chloride and anisaldehyde do not show a positive reaction to these alkaloids.

Difference from Komarowsky's Reaction

As already described, alcohols of cyclic saturated hydrocarbon, such as cholestanol and cyclohexanol, give positive results in this reaction. On the other hand, cyclohexanol shows a red coloration, when it is heated with the reagents of Komarowsky's reaction, *i.e.*, concentrated sulfuric acid and benzaldehyde. The mechanism of this known reaction was clarified by Duke,[10] who isolated cyclohexanone as an intermediate and dibenzalcyclohexanone as an end product. In order to obtain an evidence of the difference between both color reactions, the mixture of cyclohexanol, anhydrous aluminum chloride and anisaldehyde was analysed by gas chromatography. Results shown in Table Ⅶ, indicates that in this reaction cyclohexene is produced from cyclohexanol, instead of cyclohexanone. Accordingly, there is no doubt that this reaction proceeds by a mechanism different from that of Komarowsky's reaction.

Table Ⅶ. Relative Retention Times of the Reaction Mixtures

	Relative retention time		Relative retention time
Cyclohexane	1.00	Reaction mixture A[a]	1.00
Cyclohexene	1.08		0.94
Cyclohexanone	2.03	Reaction mixture B[b]	1.06
Cyclohexanol	2.54		0.94
Benzene	0.94		

a) Reaction mixture A: To 1 ml. of 5% (W/V) benzene solution of cyclohexanol, 1 ml. of 5% (W/V) nitrobenzene solution of anhydrous aluminum chloride was added, and allowed to stand at 50° for 20 min.

b) Reaction mixture B: To 2 ml. of reaction mixture A, 1 ml. of 5% (W/V) benzene solution of anisaldehyde was added at 25° and allowed to stand 25° for 20 min.
Gas chromatography——Apparatus: Barber Colman Model 20.
Column: Apiezone L capillary column, 0.2 mm.×100 feet.
Temperature: Column, 90°. Cell, 170°. Flash heater, 180°.
Carrier gas: Argon.
Carrier flow rate: 316 ml./min.
Detector: Ra-D ionization.
Retention time of cyclohexane (Standard substance): 4'36".

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Summary

A new colorimetric determination of cholesterol has been established, using anhydrous aluminum chloride and anisaldehyde as reagents. The limit and scope of the reaction on steroid was investigated, and it is concluded that a steroid having a double bond not conjugated to a carbonyl group, or having a hydroxyl group easily dehydrated gives a positive result. Availability of the reagents and difference from other known color reactions are also discussed.

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83. Takeo Naito, Renzo Dohmori, and Tatuo Kotake: Rearrangement of Sulfonamide Derivatives. V.[*1] Syntheses of Methyl α-Phenyl-2- and 4-piperidineacetate.

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In the preceding paper of this series, it was shown that the rearrangement reaction of N-acetoacetyl-2- or 4-pyridinesulfonamide 1-oxide in alkaline solution formed 2- or 4-pyridineacetic acid 1-oxide in a good yield. And these results suggested that the reaction of N-phenylacetyl-2- or 4-pyridinesulfonamide 1-oxide in alkaline solution will proceed exactly the same as in the case of N-acetoacetyl derivatives.

In the present paper, an application of this rearrangement was extended to the preparation of the medicine, methyl α-phenyl-2-piperidineacetate[*3] and its homologues.

N-Phenylacetyl-2-pyridinesulfonamide 1-oxide (Ⅱ), m.p. 190° (decomp.), was synthesized by the condensation of 2-pyridinesulfonamide 1-oxide (Ⅰ) with phenylacetyl chloride. Application of the same synthetic procedure to 4-pyridinesulfonamide 1-oxide (Ⅷ) gave N-phenylacetyl-4-pyridinesulfonamide 1-oxide (Ⅸ), m.p. 198~199° (decomp.).

The rearrangement reaction was applied to these obtained compounds.

Ⅱ evolved ammonia in 10% sodium hydroxide at 90~95°, and a resulting solution generated a strong odor of sulfur dioxide when it was acidified with hydrochloric acid. It suggested that the rearrangement reaction occurred. The reaction mixture gave colorless crystals, m.p. 102° (decomp.), whose elemental analytical values corresponded to those of α-phenyl-2-pyridineacetic acid 1-oxide (Ⅳ). In order to confirm its structure, it was decarboxylated by heating to produce a colorless needle, m.p. 101°, which was converted into a picrate, m.p. 121~122°. The elemental analytical values of these compounds corresponded to those of 2-benzylpyridine 1-oxide (Ⅴ) and its picrate respectively. In this rearrangement, Ⅱ liberated sulfur dioxide to form Ⅲ which is hydrolized to Ⅳ immediately, as reported in the preceding paper.[*1] Ⅳ gave the methyl ester (Ⅵa), m.p. 111~113° and ethyl ester (Ⅵb), m.p. 50~60°, by treatment with alcoholic hydrochloride.

N-Phenylacetyl-4-pyridinesulfonamide 1-oxide (Ⅸ) underwent rearrangement in alkaline solution to form α-phenyl-4-pyridineacetic acid 1-oxide (Ⅺ), m.p. 98~99° (decomp.),

*1 Part Ⅳ. T. Naito, R. Dohmori: This Bulletin, 3, 38 (1955).
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*3 The trade name of this compound: Methylphenidate Hydrochloride or Ritaline.